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The Effects of Carbohydrate and Protein Supplementation on Signaling Pathways Regulating Protein Turnover and Muscle Mass Following Chronic Resistance Training

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The Effects of Carbohydrate and Protein Supplementation on Signaling Pathways Regulating Protein Turnover and Muscle Mass Following Chronic Resistance Training

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Dedication

This work is dedicated to my family and friends who have supported me throughout the years. Without your support and care, I couldn't haven been standing where I am now.

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Abstract

The Effects of Carbohydrate and Protein Supplementation on Signaling Pathways Regulating Protein Turnover and Muscle Mass Following Chronic Resistance Training

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Skeletal muscle is important for physical activity and regulation of metabolism. Increase or maintenance of muscle mass is pursued by different populations ranging from athletes to people suffering from severe muscular diseases causing muscle atrophy/wasting. In this study, four animal groups were generated: sedentary group (No supplements or exercise) (SED); resistance training (RE) and whey supplements (WP); RE and combo (Carbohydrate and whey) supplements (CP); RE and placebo (DI water) (PLA). Flexor hallucis longus (FHL) muscles were collected after 8 weeks of training. Expression of several key proteins controlling muscle mass and protein turnover were measured in order to compare how different combinations affect muscle growth. It was found that resistance training induced reductions in myostatin protein expression compared with sedentary controls ($p<0.05$) and that MuRF was elevated in the CP group compared with sedentary group ($p<0.05$). We conclude that resistance training may upregulate protein synthesis through suppressing myostatin and that resistance training may increase muscle protein breakdown.

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INTRODUCTION

Forty percent of body weight is comprised of skeletal muscle, containing 50-75% of all proteins in the human body ((Rasmussen & Phillips, 2003) Skeletal muscle is important for physical activity and regulation of metabolism. Therefore, an enhancement of muscle mass is thought to be positively correlated with increased muscle strength and power, while the loss of muscle mass and strength severely affects quality of life. Athletes in power and strength sports are pursuing muscle hypertrophy to improve their performance in competitions. Meanwhile, aging, wasting of muscle use and some diseases may also lead to the quest to promote muscle hypertrophy in order to combat the loss of muscle mass and strength. Skeletal muscle constantly undergoes protein turnover and muscle growth is achieved when protein synthesis (MPS) exceeds protein breakdown (MPB), resulting in net protein accretion. These processes can be regulated through nutritional interventions and mechanical stress (Rasmussen and Phillips, 2003). Sarcopenia is the gradual loss of muscle mass usually seen as someone ages. It has been demonstrated that regular exercise combined with dietary intervention is more successful in treating sarcopenia compared with pharmacological intervention (Borster, 2004). While the effectiveness of resistance training combined with supplementations to stimulate muscle hypertrophy has been long studied, networks of signaling pathways and regulatory molecules coordinating adaptive responses to exercise have just been uncovered recently by molecular biologists (Egan and Zierath, 2013). The topic has recently received greater focus as NIH has stated “we have long known that

exercise is good for you, but no one really knows why". Achieving better health and specifically addressing the maintenance and enhancement of muscle mass has been recognized as an important topic for better daily living and sustaining functionality throughout the life span..

Resistance exercise (RE) is an important stimulus for muscle growth. It has been observed that during the initial stage of resistance training, the majority of strength gains resulted from neural adaptations (Mulligan et al. 1996). As resistance training proceeds, muscle hypertrophy occurs. It has been speculated that four primary factors are accounting for muscle hypertrophy initiated by resistance exercise: mechanical tension, muscle damage and recovery time and appropriate nutrition (Schoenfeld, 2010).

Resistance exercise elevates mechanical stress upon the muscle, disturbs the integrity of muscular tissue. The mechanical tension is transduced through downstream signaling pathway such as IGF-1/PI3K/Akt/mTOR to promote protein synthesis. It has been demonstrated that hypertrophy associated with mechanical contraction can also be achieved by activating mTOR through phosphatidic acid independent of IGF-1/PI3K pathway (Hornberger et al. 2006). Protein synthesis can be regulated through two signaling pathways, the IGF-1-Akt-mTOR pathway, acting as a positive regulator, and the myostatin pathway, acting as a negative regulator. Ruas and his colleagues have shown that PGC1-alpha 4 is a unique transcription co-factor modulating IGF-1 and myostatin. In concert these factors promote muscle hypertrophy by upregulating IGF-1 while suppressing myostatin. Resistance training has been demonstrated to increase expression of PGC1-alpha 4 and reduce expression of myostatin (Ruas et al. 2012).

However, more research is necessary to uncover the underlying mechanisms as to how PGC1-alpha 4 mediates muscle growth. Exercise training can also induce localized damage, stimulating inflammatory factors, and subsequent regenerative factors to generate a hypertrophic response through activation of satellite cells via upregulation of mechano growth factor (Hill and Goldspink 2003). There're some weak points on the sarcomeres, which are vulnerable to lengthening, and may cause a shearing of myofibrils when subjected to repeated movements. This muscular trauma is associated with inflammatory response, in which macrophages and neutrophils migrate to the injury site and remove the debris. Subsequently, these macrophages undergo polarization from a pro-inflammatory state to a pro-regenerative state, releasing cytokines and growth factors that further mediate satellite cell activation and muscle growth (Toigo and Boutellier, 2006; Vierck et al. 2000). Moreover, metabolic stress results from exercise that mainly relies on ATP/CP or anaerobic glycolysis is partially responsible for exercise-induced muscle hypertrophy, as these energy systems lead to the metabolite buildup such as lactate acid, hydrogen ion and others (Suga et al. 2009). The metabolic stress results in alterations in hormonal milieu and free-radical production, inducing increase in muscle mass (Gordon et al. 1994; Goto et al. 2005).

Nutrient availability is one of the principal determinants of skeletal muscle protein turnover. It has been shown that in the early stages of recovery from exercise, muscular protein breakdown (MPB) may exceed muscular protein synthesis (MPS) in the absence of nutritional intake (Biolo et al. 1997). Transfer and incorporation of amino acids captured from dietary protein sources into skeletal muscle proteins is how

protein supplementation functions to demonstrate its anabolic effects (Atherton and Smith 2012). Since muscle protein is lost in fasted periods due to amino acid oxidation and gluconeogenesis, there's a need to compensate for the loss (Wackerhage and Rennie, 2006). Therefore, protein supplementation with resistance exercise potentiates muscle protein accretion by supplying the amino acids pool. It's been shown that protein supplementation can also positively regulate mRNA translation initiation via activating the mTOR signaling pathway and that increase of protein synthesis in response to increased essential amino acids will be blocked upon inhibition of mTOR (Dickinson et al. 2011). With protein or amino acid (AA) feeding, there's a transient but significant increase in the rate of MPS without much change in MPB, rendering a positive net protein balance (Biolo et al. 1997). Three most commonly consumed protein sources are soy, casein and whey. Whey is rich in leucine and can be digested relatively fast and induce a more transient but larger increase in MPS compared with casein (Tang et al. 2009). It was also found that within 3h post-exercise, whey protein could stimulate the highest rise in MPS compared with the other two sources (Tang et al. 2009). Protein supplementation in conjunction with resistance training is also associated with increases in serum insulin and IGF-1, both of which are able to augment MPS (Ballard et al. 2005). Insulin and IGF-1 can act on the insulin receptors located on sarcolemma, stimulating the PI3K-Akt/mTOR signaling pathway, which, as aforementioned, can positively affect MPS (Bolster et al. 2004). Besides, leucine is known to positively affect the downstream factors of mTOR pathway, indicating that AA and insulin function synergistically to optimize anabolic response in skeletal muscle (Rasmussen et al. 2000). It's noteworthy

that provision of protein alone (without carbohydrate) causes a rise in insulin secretion similar to a mixed meal (Artherton et al. 2010). Besides, there's an elevation in MPS upon infusion of amino acids even when insulin is clamped with β-cell inhibitor (Greenhaff et al. 2008). However, it has been shown that insulin plays an important role in an anti-catabolic fashion, in which it can suppress MPB. Question remains as to if taking carbohydrate on top of protein is necessary in order to get optimal muscle growth. At rest, insulin, when infused along with AAs, can promote MPS and suppress MPB (Bennet et al 1990). Biolo also demonstrated that following resistance exercise (RE), insulin is able to attenuate MPB without much impact on MPS (Biolo et al.1999). It's reasonable to speculate that carbohydrate ingestion may potentiate muscle growth by inducing insulin secretion. However, while insulin is able to increase MPS upon activation of mTOR pathway, when AA delivery are increased as a result of protein/AA supplementation, it seems that AA alone can induce secretion of insulin and even low levels of insulin are able to optimize the stimulation. Therefore, co-ingestion of protein/AA with carbohydrate may not be able to further stimulate MPS compared with sole ingestion of protein/AA as long as protein/AA is adequate. (Huang & Manning, 2008)Koopman and his coworkers found that co-ingestion of carbohydrate with protein didn't further augment postexercise MPS (Koopman et al. 2007), validating the aforementioned mechanism. However, there remain valid reasons to incorporate carbohydrates into protein/AA supplements following resistance exercise, to replenish glycogen depletion and to suppress MPB.

Thus, this study will investigate how different supplements combined with resistance training affect muscle hypertrophy and through what mechanisms.

LITERATURE REVIEW

MYOGENIC PATHWAYS AND MUSCLE HYPERTROPHY

The maintenance of muscle mass is important, because both sedentary and active adults will lose 30-40% of their muscle mass by the age of 80, which will severely affect their quality of life due to loss of independence and increased risk of injury. Meanwhile, an increase in muscle mass is desired by athletes to improve their performance in various competitions. Since muscle mass is a balance of muscle protein synthesis and muscle protein breakdown, the development of appropriate strategies to accelerate MPS and reduce MPB to maintain or even increase muscle mass is of great clinical and athletic importance. However, in order to develop such strategies, we must first need to establish a comprehensive understanding of the molecular mechanisms that regulate muscle mass/protein turn over.

Anabolic Pathways

Akt/mTOR

In adult skeletal muscle, the Akt/mTOR signaling pathway is currently believed to act as the major pathway regulating skeletal muscle growth (McCarthy and Esser, 2010). In general, Akt is a molecular nodal point, served as both an effector of anabolic signaling and an inhibitor of catabolic signaling. Research over the past years have revealed that mammalian target of rapamycin complex 1(mTORC1), here referred to as mTOR, is a central component of a signaling network that controls muscle mass, which can be regulated by various factors, including growth hormones, nutrients and mechanical stimuli. The mTOR complex consists of the proteins mTOR, G protein beta-

subunit-like protein, and regulatory protein Raptor (Huang & Manning, 2008). The mTOR protein is a Ser/Thr kinase that contains multiple domains including FKBP12-rapamycin binding domain (FRB), which will exert inhibitory effects on mTOR signaling when bound to FKBP12-rapamycin complex. The Raptor protein is an adaptor protein, recruiting downstream substrates to bind with mTOR and get phosphorylated. IGF-1 is well characterized as one of the growth hormones that trigger muscle(Rommel et al., 2001) hypertrophy through various mechanisms, including activating PI3K/PKB-Akt-mTOR pathway, mediating proliferation and differentiation of satellite cells and enhancing the fusion of satellite cells with existing muscle fibers to maintain the nuclear domain. Upon mechanical loading, it is widely accepted that mechanical stimuli activate mTOR through IGF1-mediated mTOR pathway, since muscles produce more IGF-1 through both paracrine and autocrine manner during mechanical stimulation (Rommel et al., 2001) and the subsequent constitutive activation of PI3K-PKB pathway induces mTOR activation. IGF-1 activates class I phosphoinositide -3 kinase (PI3K), which further phosphorylates protein kinase B (PKB) (Reiling & Sabatini, 2006). Activated PKB inhibits the activity of the tuberous sclerosis complex (TSC1/2) complex, which acts as a GTPase for a protein named homolougously enriched in brain (Rheb). Rheb is a molecule that interacts with mTOR, which activates mTOR kinase domain and promotes the activation of mTOR signaling when charged with GTP (Huang & Manning, 2008). However, recent reports have shown that IGF-1 may not be absolutely necessary for the induction of muscle hypertrophy and other upstream mediators activating mTOR signaling may be involved to dictate muscle hypertrophy. A study conducted by

Spagenburg et al. demonstrated that mechanical activation of mTOR and hypertrophic response were still preserved with a transgenic mouse model lacking a functional IGF-1 receptor, providing evidence that other upstream substrates may be involved in this process (Spagenburg et al. 2008). It has also been found that mechanical activation of mTOR can be independent of IGF-1 and actually acts through phosphatidic acid (PA), which competes with FKBP12-rapamycin complex to bind to the FRB domain site on mTOR to confer the resistance to the inhibitory effects that ramapacin has on mTOR (Hornberger et al. 2006). Furthermore, work from the Hornberger laboratory extends these initial findings by reporting that eccentric contraction-induced activation of mTOR was independent of PI3K-Akt activity (O'Neil et al. 2009). Thus, both IGF-1 and PA can be upstream triggers of mTOR signaling.

Once mTOR is activated by phosphorylation, it will further activate other downstream factors like 70 kDa ribosomal protein S6 kinase (70S6K), which is typically used as a read-out of mTOR signaling. Baar and Esser found that the magnitude of the increase in the phosphorylation of 70S6K was highly correlated with the hypertrophic gains of muscles subjected to resistance exercise (Baar & Esser, 1999). The p70S6K is thought to induce the phosphorylation of ribosomal protein S6 (rpS6), one of the components of the eukaryotic 40S ribosomal subunit (Jastrzebski et al. 2007). Upon phosphorylation, rpS6 can further dictate the translation of mRNAs involved in increasing protein synthesis.

β-catenin/c-Myc signaling

Mechanical stimuli regulate protein synthesis through changes in both translational efficiency and translational capacity, the former one of which consists three main steps: initiation, elongation and termination. The pathway mentioned above is revealed to be primarily involved in the stage of initiation(Kimball, Farrell, & Jefferson, 2002). β-catenin/c-Myc signaling pathway is considered to affect translational capacity, an important factor determining MPS and defined as the total ribosomal content per unit tissue (McCarthy and Esser 2010). Studies have provided evidence, suggesting that β-catenin/c-Myc signaling pathway operates independent of the mTOR pathway in regulating ribosomal biogenesis. Mechanical overload-induced muscle growth was completely prevented by the muscle specific inactivation of the β-catenin gene, indicating the necessity of β-catenin for muscle hypertrophy.

Catabolic Pathways

Muscle mass regulation is a sum of MPS and MPB, the latter of which involves three principal proteolytic systems in skeletal muscle: the ubiquitin-proteasome system, the autophagy-lysosome system and the Ca2+-dependent calpains and caspases. The ubiquintin-proteasome system is required to mediate myofibrillar protein degradation upon changes in muscle activity such as mechanical loading or unloading. The proteasome complex can only recognize and remove proteins with ubiquitins attached to them. There are three types of ubiquitin ligases believed to be involved in this process: the uniquitin-activating enzyme (E1), conjugation enzymes (E2s), and specialized ligases (E3s). Different E2-E3 pairs will dictate different proteins to be degraded and is precisely regulated. Goldberg's group made a major contribution to identify the specific ubiquitin

ligases involved in the loss of muscle mass by comparing gene expression in different models of muscle atrophy(Gomes, Lecker, Jagoe, Navon, & Goldberg, 2001). The genes induced most among these diverse disease models were found to encode two crucial ubiquitin E3 ligases in the skeletal muscle, atrogin-1 (also known as muscle atrophy F-box (MAFbx)) and muscle ring-finger protein 1(MuRF1). The substrates for atrogin-1 seem to be involved in growth-related processes, such as MyoD, a key muscle transcription factor associated with satellite cell differentiation, while MuRF1 was reported to control muscle structural proteins, such as troponin I and myosin heavy chains (Kedar et al., 2004). Autophagy is considered as a non-selective degradation pathway compared to ubiquitin-proteasome system. Lysosomes are membrane-bound organelles that can degrade proteins delivered to them as autophagic cargo. The crucial role of autophagy-lysosome system in skeletal muscle is recognized by evidence showing that alterations in this process lead to several genetic muscle diseases. Too much autophagy impairs myofiber homeostasis, causing excessive removal of proteins that are needed to maintain the normal cellular activities. On the other hand, insufficient autophagy results in retention of damaged components, leading to muscle weakness. Ca2+-activated proteasomes like calpains were thought to be involved in the degradation of contractile proteins (Purintrapiban et al. 2003). Several signaling pathways have been recognized recently to play important roles in muscle protein degradation and will be discussed separately below.

NFkB sinaling

NFkB transcription factors can mediate the effect of inflammatory cytokines such as tumor necrosis factor-alpha (TNFa) on muscle wasting ((Kang, Goodman, Hornberger, & Ji, 2015). When inactivated, NFkB is sequestered in the cytoplasm by inhibitory factors IkB. The IkB kinase (IKKb) complex phosphorylates IkB in response to TNFa, leading to IkB's degradation, which initiates the translocation of NFkB to induce NFkB-mediated gene transcription like MuRF1 (Peterson, Bakkar, & Guttridge, 2011) Numerous studies have clearly shown the role NF-κB signaling plays in muscle atrophy. By introducing the dominant-negative inhibitor of κBα to inhibit the activity of NF-κB, Andrew Judge and coworkers prevented muscle fiber atrophy with contractile claudication in patients with peripheral arterial disease (PAD) (Hain, Dodd, & Judge, 2011). Furthermore, the Judge laboratory has reported that heat shock protein 70 (Hsp70) and Hsp27 can prevent the loss in fiber cross sectional area (CSA) with immobilization and inhibit NF- κB activation (Senf et al. 2008; Dodd et al. 2009).

Akt/FOXO3A signaling

MAFbx and MuRF1 are muscle-specific E3 ubiquitin ligase genes, which are thought to be central components of the ubiquitin/proteasome system in skeletal muscle. FOXO3A transcription factors can initiate muscle atrophy by activating MAFbx and MuRF1. FOXO3A is trapped in the cytoplasm when inactivated upon phosphorylation by Akt. However, once dephosphorylated, FOXO3A can enter the nucleus and induce the expression of atrogin and MuRF1, causing muscle atrophy. As mentioned above, IGF1 can elevate MPS by activating PI3K-Akt-mTOR-70S6K-rpS6 pathway. Akt can control both protein synthesis, via mTOR, and protein degradation, via

FOXO3A. Several studies demonstrated that upregulation of atrogin and MuRF1 was blocked by Akt through exporting FOXO3A from the nucleus to the cytoplasm (citations, Lee et al, 2004). FOXO has also been found to interact with PGC-1alpha, a critical coactivator for mitochondrial biogenesis. Kang et al. revealed that overexpression of PGC-1alpha via transfection ameliorated mitochondrial deterioration and accelerated muscle mass recovery during the remobilization period following 14 weeks of immobilization in mice, with concomitant reduction of active FOXO3A, atrogin and MuRF1 (Kang et al. 2015).

Myostatin Signaling

Myostatin is a member of TGFbeta family, predominantly expressed and secreted by skeletal muscle, functioning as a negative regulator for muscle growth. Mutations of the myostatin gene have led to excessive muscle growth observed in mice, sheep and cattle. It was demonstrated by recent studies that myostatin regulates muscle mass through two transcription factors, Smad2/Smad3. Interaction with Activin A allows myostatin to bind to and activate a heterodimeric receptor complex with kinase activity, comprising a type II receptor, activin receptor 2 (ActRIIB) and activin type I receptor, activin receptor-like kinase 4 and 5 (ALK4/5). Activation of activin type I receptor leads to the phosphorylation of Smad2/3, of which phosphorylated forms result in the formation of a heterodimeric complex with the common mediator Smad4 (Rodriguez et al., 2014; Schiaffino, Dyar, Ciciliot, Blaauw, & Sandri, 2013). However, the transcriptional targets of the heterodimeric complex have still been under investigation to uncover the inhibitory effects of myostatin on mucle growth.

A series of studies have suggested an interaction between myostatin signaling and Akt/mTOR signaling in regulating muscle mass (Sartori et al. 2009; Amirouche et al. 2009). Activation of TGF-β through myostatin treatment has been shown to be associated with decreased activation of Akt as well as other components of Akt/mTOR signaling such as ribosomal protein S6, p70S6K (Trendlenberg et al. 2009). Inhibition of myostatin resulted in muscle hypertrophy dependent of mTOR signaling and independent of MuRF1 (Sartori et al. 2009). It seems that there's some degree of crosstalk between Akt/mTOR and TGF- β pathways. However, other studies have revealed that myostatin activates FOXO1, leading to increased expression of atrogin in muscle cell cultures (McFarlane et al., 2006). Besides, myostatin expression is found to be under the regulation of FOXO1, which further supports the idea that myostatin pathway interacts with Akt/FOXO signaling (Allen & Unterman, 2007).

MUSCLE ADAPTATION TO CHRONIC RESISTANCE TRAINING

Muscle adaptation and protein turnover in response to resistance training

In humans, provided sufficient exercise intensity, acute increases in the rates of MPS and MPB will occur (Phillips et al. 1997). A single bout of resistance exercise can upregulate gene expression of several factors associated with muscle growth, indicating that muscle hypertrophy occurring with frequent bouts of resistance exercise can be partially explained by translational mechanisms (Willoughby and Nelson 2002). Animal studies can provide important information in terms of unraveling complicated cellular and molecular mechanisms. Therefore, mechanisms explaining muscle

hypertrophy through resistance training, different training modes as well as some animal models developed to mimic resistance training will be briefly reviewed below.

Mechanisms of Resistance Exercise-Induced Muscle Hypertrophy

Chronic resistance training can primarily lead to muscle hypertrophy, an increase in muscle cross-sectional area (CSA). Increased MPS allows for expansion of myonuclear domain, which is the area governed by a single muscle nucleus. However, it's considered that a ceiling exists for myonuclear domain and further increase in muscle mass requires the infusion of new nuclei to the muscle fiber to provide sufficient cytoplasmic capacity for protein expansion to occur, which suggests activation of satellite cells. Normally, satellite cells are quiescent and reside between basal lamina and sarcolemma. However, these myogenic stem cells can be activated and will proliferate, differentiate and fuse with existing cells or among themselves to create new myofibers during muscle regeneration or when there is a sufficient mechanical stimulus imposed on muscle fibers (Vierck et al. 2000). Therefore, hypertrophy could conceivably result from either an increase in the number of domains or an increase in the size of existing domains (Edgerton and Roy, 1991). The former concept has been strongly supported by research to date. Irradiation of satellite cells preceding TA muscle ablation has been demonstrated to block the hypertrophy of EDL muscle (Rosenblatt et al. 1994). Therefore, satellite cells may serve as the pool of a myonuclei to support muscle growth. Besides, satellite cells co-express myogenic regulatory factors like Myf5, MyoD, which are thought to play distinct roles in myogenesis (Sabourin and Rudnicki 2000). However, satellite cells will only be activated when the myonuclear domain reaches a critical size, below which accumulation of protein seems to be the sole factor accounting for muscle hypertrophy.

The fact that MPS exceeds MPB following acute resistance exercise is well documented in both animals and humans, and these acute anabolic effects are believed to responsible for muscle growth over time during a longer training period. However, chronic resistance exercise (RE) training appears to attenuate the acute RE induced MPS . This can be partially explained by the adaptive muscle not stressed efficiently by the same absolute workload. Another reason accounting for this phenomenon is that training may shift the diurnal pattern of MPS. The latter explanation was supported by a study conducted by Gasier et al, where they found no significant difference in the acute rates of MPS (16h) between trained rats and sedentary controls, while the cumulative fractional rates of MPS (36h) did appear to be higher in the plantaris of trained rats cmpared to sedentary controls. This suggests that trained individuals may have a more rapid and stronger increase in the rate of protein synthesis. As mentioned above, the molecular mechanisms behind the elevation of MPS is the activation of mTOR, which can be either achieved by phosphorylation of Akt or binding with PA. Recent studies have revealed that a novel form of PGC-1alpha (PGC-1alpha4), which results from alternative splicing of the primary transcript, is also involved in muscle growth. It was shown that myotubes transduced with PGC-1alpha4 with adenovirus stimulated IGF-1 expression and suppressed myostatin expression, resulting in a 2-fold increase of protein/DNA compared with normal myotubes. In a hindlimb suspension model, they found that the expression of PGC-1alpha 4 decreased during the unloading phase and increased after reloading with a concomitant increase in the expression of IGF-1 and reduction of myostatin. Myotube growth induced by treatment with clenbuterol was blunted by PGC-1lapha4 knockdown

(Ruas et al. 2012). All of the evidence suggests a crucial role PGC-1alpha4 plays in regulating muscle mass

Resistance Training Modes

Resistance training has been well documented as a stimulus for muscle growth. However, it's still controversial as to which exercise program is the best to trigger muscle hypertrophy. It has been proposed that reaching contractile failure is required for optimal muscle growth regardless of training loads (Morton et al. 2015). This contradicts the training protocol pointing out that relatively high training loads are better compared with low training loads to induce muscle growth (Campos et al. 2002). It has been suggested that as long as muscle fatigue exists, some motor units will drop out, requiring new motor unit activation and recruitment of other muscle fibers, which can probably result in a similar degree of muscle growth in either low- or high- resistance training (RE), considering comparable motor units being activated till contractile failure (Morton et al. 2015). However, further studies are needed to compare the two different exercise approaches and find out the "best" way to optimize muscle hypertrophy.

Animal Models

Several animal models have been established to mimic human resistance training. Rats were stimulated to perform human squats. Squat training was composed of high-intensity, short duration and graded overload exercise, which is very similar to the progressive overload exercise fashion that human goes through. 12 weeks of training resulted in fiber hypertrophy in the squat group compared with control, indicating that squat protocol is a good surrogate of RE for animals to induce muscle hypertrophy (Tamaki et al. 1992). However, changes in muscle mass was only observed when

expressed as a ratio of muscle weight to body weight, there was no absolute gains in muscle mass. Therefore, another animal model was developed, i.e. ladder climbing, to mimic the training parameters and adaptations observed in human progressive RE. After 8 weeks of RE, it was shown that there was a 23% absolute increase in flexor halluces longus (FHL) muscle mass, with a daily accretion of about 0.3% (Hornberger and Farrar 2004).

NUTRIONAL INTERVENTION ON RESISTANCE TRAINING

Protein supplementation on muscle protein turnover

High intensity of resistance training can cause a weaker training response and a prolonged recovery period with insufficient nutrient supplementation. It has been shown that in the early stages of muscle recovery from resistance exercise, MPB may exceed MPS in the absence of nutritional intake (Biolo et al. 1997). However, protein supplementation can favor MPS and augment protein accretion. Protein can increase the availability of amino acid for the muscle, thereby providing sufficient substrate for MPS (Bohé, Low, Wolfe, & Rennie, 2001). A meta-analysis performed by Cermak et al. summarized that the dietary protein supplementation increased fat free mass and strength gains compared with a placebo after prolonged resistance exercise training (Cermak et al. 2012). It has also been shown that 10 weeks of resistance training in conjunction with either whey protein supplementation or dextrose placebo resulted in upregulation of markers indicative of MPS and subsequent improvements in muscle performance with protein supplementation being more effective than carbohydrate (Willoughby et al. 2007). Another study conducted by Stine et al. also demonstrated that whey protein plus

carbohydrate on top of RE augmented muscle hypertrophy compared to isocaloric carbohydrate supplementation only (Rahbek et al., 2014). While they observed elevated phosphorylation of mediators involved in MPS, such as mTOR, Akt and 70S6k during acute RE, they failed to detect any changes in the total protein levels of Akt-mTOR pathway proteins following prolonged intervention, which suggests that these anabolic signaling proteins promote MPS mainly through acute phosphorylation. Leucine has been found to cause a high insulin response and believed to interact with intracellular sensors that activate mTOR (Dibble & Manning, 2013). It is believed that amino acids regulate mTOR through a different mechanism other than the class I PI3K-PKB pathway. However, it was also observed that activation of mTOR by nutrients was blocked when introducing the PI3K inhibitor wortmannin, suggesting that PI3K is required for this event. This led to the finding of a wormannin-sensitive class III PI3K named Vps34, which is now thought to be involved in AA-induced mTOR activation. The most widely accepted mechanism of AA-induced activation of mTOR involves the translocation of mTOR to lysosomal membranes, where both Rag GTPases and Rheb reside (Sancak et al. 2010). Protein supplementation in conjunction with resistance training is also associated with increases in serum insulin and IGF-1, both of which are able to augment MPS (Ballard et al. 2005). Insulin and IGF-1 can act on the insulin receptors located on sarcolemma, stimulating the PI3K-Akt/mTOR signaling pathway, which, as aforementioned, can positively affect MPS (Bolster et al. 2004).

Caborhydrate supplementation on muscle protein turnover

The most acceptable mechanism for carbohydrate supplementation induced elevated MPS is via its positive effects on insulin release. At rest, insulin, when infused along with AAs, can promote MPS and suppress MPB (Bennet et al 1990). Other study found that following RE, insulin is able to attenuate MPB without much impact on MPS (Biolo et al.1999). While insulin is able to increase MPS upon activation of mTOR pathway, when AA delivery are increased as a result of protein/AA supplementation, it seems that AA alone can induce secretion of insulin and even low levels of insulin are able to optimize the stimulation. Therefore, co-ingestion of protein/AA with carbohydrate may not be able to further stimulate MPS compared with sole ingestion of protein/AA as long as protein/AA is adequate (Robert et al. 2015). Koopman and his coworkers found that co-ingestion of carbohydrate with protein didn't further augment postexercise MPS (Koopman et al. 2007), validating the aforementioned mechanism. However, a study conducted by Bird et al. has revealed that carbohydrate (CHO) and essential amino acids (EAA) supplementation augments the anabolic response and hypertrophic gains compared to CHO, EAA or placebo group presumably by attenuating the post-exercise rise in protein degradation (Bird et al. 2006). Elevated cortisol levels post exercise confirms that catabolic events may predominate in the early stage post-exercise recovery from RE. Reduction of insulin levels will impede MPS, because insulin is one of the growth factors that can dictate MPS through activating mTOR signaling pathway. It was demonstrated that CHO+EAA supplementation potentiated the stimulation of MPS induced by ingestion of EAA alone in the presence of acute hypercortisolemia. EAA and CHO supplementation can increase the AA availability and insulin release, which

suppresses exercise-induced cortisol secretion, and therefore can possibly optimize muscle growth by enhancing MPS and suppressing MPB. However, as the results from various studies are controversial, there still remains a big gap as whether and how addition of CHO to protein supplementation can augment protein accretion during a long term period of RE.

SIGNIFICANCE

Comprising 40-50% of the total body mass, skeletal muscles are not only motors that drive locomotion, but they also play critical roles in breathing and whole body metabolism. Therefore, loss of muscle mass and strength with aging or disease severely affects quality of life. Both sedentary and active adults will lose 30-40% of their muscle mass by the age of 80, leading to their loss of independence, increased risk of morbidity and mortality, as well as an estimated $18.5 billion in annual healthcare costs in the United States. On the other hand, Athletes in power and strength sports are pursuing muscle hypertrophy to improve their performance in competitions. Thus, the development of appropriate strategies or therapies to restore, maintain and even increase muscle mass is of great importance both clinically and fiscally. Sarcopenia is the gradual loss of muscle mass usually seen as someone ages. It has been demonstrated that regular exercise combined with dietary intervention is more successful in treating sarcopenia compared with pharmacological intervention (Borst 2004).

Acute RE elevated both MPS and MPB. Protein supplementation may increase MPS with minor effect on MPB. Recent research has suggested that addition of CHO to protein supplementation may potentiate muscle mass increase by inhibiting MPB via suppressing cortisol levels. However, the studies yielded controversial results in terms of the additive effects that CHO has on MPS. While the effectiveness of resistance training combined with supplementations to stimulate muscle hypertrophy is long known, networks of signaling pathways and regulatory molecules coordinating adaptive responses to exercise have just been uncovered recently by molecular biologists (Egan

and Zierath, 2013) and is still being largely investigated. Therefore, the goal of this study was to develop more effective ways to optimize muscle hypertrophy and understand the underlying mechanisms to shed some light upon possible therapeutic targets to promote increase in muscle mass.

METHODS

ANIMAL

A total of 31 male Sprague-Dawley rats were obtained at approximately 2 months of age from Charles River (Wilmington, MA). The rats were housed individually in their cages in order to monitor their diets and were maintained on a 12-hour light/dark cycle with the light phase from 8:pm to 8:am. Rats were provided standard lab chow and allowed ad libitum access to water. All experimental procedures were approved and conform the guidelines set by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas at Austin.

EXERCISE FAMILIARIZATION

Following 1 week of acclimation to their new environment, rats underwent three repeated sessions of ladder climbing separated by 1 day between each session to get familiarized with the exercise protocol. During the familiarization period, rats carried no weights and climbed a ladder 1 m in height on an incline of 85° with 2 cm grid steps. Rats then completed three practice sessions of climbing separated by 1 day between each session with 50, 60, and 70 % of their body mass attached to their tails, respectively. The weight was attached at the base of the tail with foam tape (3 M Conan) and a Velcro strap. Rats were encouraged to climb by lightly tapping their tails with a bottlebrush.

EXPERIMENTAL PROTOCOL

During the experimental procedure, rats began high-intensity progressive resistance training according to Hornberger and Farrar (Hornberger and

Farrar, 2004). Following a 3-hour fast, rats were trained to climb the ladder once every three days for 8 weeks. Each training session consisted of 4-9 climbs requiring 8-12 dynamic movements per climb. Based on the performance, the weight carried during each session was progressively increased.

Rats began climbing with carrying a weight equal to 75 % of their body mass attached at the base of the tail. There was a 2-min rest period between each climb. Upon successful completion of this load, an additional 30 g weight was added to the load apparatus. This procedure successively repeated until a load was reached with which prevented the rat from climbing the entire length of the ladder. The highest load successfully carried during the initial training session was considered as the rat's maximal carrying capacity for that training session. Subsequent training sessions consisted of 4-9 ladder climbs. During the subsequent first 4 ladder climbs, the rats carried 50%, 75%, 90% and 100% of their previous maximal carrying capacity, respectively. Then an additional 30g load was progressively added until the rat's new maximal carrying capacity was achieved. This training regimen was repeated once every 3 days for 8 weeks, a total of 20 training sessions. Food consumed was be recorded every day.

When every training session was completed, whey hydrolysate (WP = 0.5g/kg), carbohydrate plus whey (CP: whey=0.5g/kg, CHO=1.2g/kg) or placebo (8 ml/kg DI water) was given immediately post resistance exercise by intubation. Following intubation, lab chow was removed from cages for three hours and rats were allowed access to water ad libium. A total of seven rats were used as sedentary controls and

received an intubation of DI water (8 ml/kg) at the same time when rats from other groups were gavaged.

Rats in each treatment group were subdivided by time of euthanasia, which occurred within 24 hours post intubation (n = 7-8 per group). Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital, (75 mg/kg of body weight) during which the flexor hallucis longus (FHL) were excised, freeze clamped in liquid nitrogen, and stored at – 80 °C for later analysis by Wendy Wang. Rats were then euthanized by cardiac injection of sodium pentobarbital (65 mg/kg of body weight).

WESTERN BLOT ANALYSIS

The frozen muscle samples weighing ~100 mg were homogenized in ice-cold homogenization buffer [50 mM HEPES (pH 7.6), 150mM NaCl, 1% Triton-X 100, 20mM beta-glycerol phosphate, 10mM NaF, 100 1 mM Na3VO4, 50 mM glycerophosphate, 10ng/ml each of leupeptin and aprotinin, 1 mM phenylmethanesulphonyl fluoride (PMSF), and 1:100 dilutions of phosphate inhibitor cocktails 1&2 (Sigma-Aldrich).] at a 1:15 dilution of wet weight muscle. The resulting homogenates was centrifuged at 12,000g for 30 min at 4 °C, and the supernatants were taken for measurement of total protein concentration determined as described by Bradford (74, David's dissertation). All muscle homogenate aliquots were then be stored at –80 °C until analysis. Samples were boiled in 4X Laemmli's sample buffer at a ratio of 3:1 at 95 °C for 10 min in order to denature muscle, and equal amounts of total protein were loaded into each well of a 5% stacking/12.5%-15% separating polyacrylamide gel. Gels were run at constant current of 50mA for about 70 min until necessary protein

separation was achieved (Bio-Rad Laboratories, Hercules, CA.). The resolved proteins were then electrically transferred onto a PVDF membrane (Millipore) using a semi-dry transfer unit (Bio-Rad Laboratories, Hercules, CA) at 300mA for 45 min. Ponceau S. (0.1 % in 0.5 % acetic acid) was used to verify the completeness of the transfer. The membranes were then washed in Tris-buffered saline (TBS) with 0.06 % Tween20 (TTBS) to remove the Ponceau S staining, and then the membranes were blocked with 5% skim milk in 0.06%TTBS (blocking buffer) for 1 h at room temperature (RT). The membranes were then incubated with the appropriate primary antibody overnight at 4 °C, usually in 1:1000 dilutions of primary antibody in either 1% skim milk-TBST or 5% BSA-TBST. The targeted proteins are PGC1-alpha4/1, myostatin, Murf, Atrogin, Foxo3a and phosphorylated Foxo3a. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used as an internal loading control. Most of the antibodies were purchased from Cell Signaling Technology (Cell Signaling Technology, Beverly, MA), except that the primary antibody for Gapdh was purchased from Santa Cruz and the primary antibody for Foxo3a was purchased from abcam. Following overnight primary antibody probing, all membranes were washed 5 min, three times with TTBS. Then, the membranes were incubated with a dilution of 1:2000 HRP-conjugated secondary anti-rabbit, anti-mouse or anti-goat IgG (Pierce) in 1% skim milk-TBST for 1 hour, depending on the host of the primary antibodies. After three additional 5-min washes, the membranes were visualized by enhanced chemi-luminescence (ECL) in accordance with the manufacturer's instructions (Perkin Elmer, Boston, MA). All western blots were performed in duplicate for each muscle sample to ensure reproducibility. Images were captured using a charge-

coupled device camera in a Chemi- Doc system (Bio-Rad, Hercules, CA). Intensity of each band was quantified with Image Lab analysis software (Bio-Rad). Following ECL detection of phosphor-proteins, membranes were stripped and re-probed for total protein or internal controls.

STATISTICAL ANALYSIS

All values are expressed as mean ± SEM. A one-way ANOVA was used for the western blot results, and Fisher's LSD post hoc test was performed to compare mean differences among treatments. Differences with p-values < or = 0.05 were considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics v19.0 software (IBM Corporation, Armonk).

RESULTS

Expression of proteins involved in the regulation of muscle mass and protein degradation was investigated using western blot. PGC-1alpha isoform 1 and 4 levels are not different among groups. However, there is a trend of decline in PGC-1alpha1 and increase in PGC-1alpha 4. Myostatin protein levels are significantly lower in exercise groups compared to sedentary groups ($p < 0.05$). Neither p-FOXO3aA nor FOXO3A demonstrates to be different among groups. However, pilot data using the ratio of p-FOXO3A/total FOXO3A shows a trend of reduction in this ratio upon mechanical stimulation via resistance training. MuRF protein expression is significantly greater in CP group than that in sedentary group ($p<0.05$) and no differences exist among other groups. There is no significant difference in the expression of Atrogin across groups. However, atrogin levels tends to be higher following resistance training.

DISCUSSION

The primary findings of the present study was that myostatin levels, a negative regulator of muscle mass decreased upon resistance training. MuRF, known as one of the skeletal muscle specific E3 ubiquitin ligases, increased after resistance training when supplemented with carbohydrate and protein compared with sedentary control. Previous analysis conducted by Wang et al. (unpublished data) demonstrated that following 8 weeks of resistance training, the gains in the maximal capacity were greater in the CP group compared with either PLA or WP groups (Figure 4). Although it was shown by Wang et al that resistance training did increase the FHL muscle mass, fiber CSA and muscle CSA when compared to sedentary control (Figure 3), which was indicative of muscle hypertrophy, there were no significant differences among training groups, suggesting that supplementations didn't seem to further increase protein accretion. Total and myofibrillar protein concentration and content were then measured by Wang et al. and the content of myofibrillar protein was shown to be greater in the exercise groups than sedentary controls, demonstrating that resistance training induced myofibrillar protein accumulation. It was worth noting that myofibrillar protein content was greater for CP than for WP group and trended to be higher than PLA group, accounting for the higher increase in maximal capacity (Figure 2).

The net balance of protein synthesis and protein breakdown will dictate protein turnover and muscle mass. It was demonstrated by Wang et al. that the total protein content was elevated significantly in CP group and slightly elevated in WP and PLA groups compared with sedentary controls. Together with the CSA and mass data, it is

speculated that resistance training in the present study stimulated muscle protein synthesis. It was found by Wang et al. that IGF-1 protein expression increased only in the CP group compared with SED group (Figure1). In our study, we measured myostatin protein expression and found that to be down regulated significantly upon resistance training (Figure 5). IGF-1 acts as a stimulus for protein synthesis, functions through an autocrine/paracrine fashion. Upon mechanical stimulation, there's a systemic elevation of IGF-1 as well as a higher utilization of circulating IGF-1 by the skeletal muscle (Goldspink, 2005), which allows a greater likelihood of IGF-1 to bind with IGF binding proteins, facilitating protein synthesis through PI3K-PKB-Akt-mTOR pathway. Myostatin, on the other hand, is thought to be negatively affecting protein synthesis by interacting with Akt/mTOR pathway. Kim et al. conducted a study investigating the impact of resistance loading on myostatin expression in different gender and age populations and found that resistance loading induced declines in myostatin mRNA transcripts in most of the populations(Kim, Cross, & Bamman, 2005). Another study conducted by Roth et al. also demonstrated that 9-week heavy resistance loading reduced myostatin mRNA levels ((Roth et al., 2003). However, in contrast to our findings and to those of Roth et al. and Kim et al., Willoughby reported increased levels of myostatin protein and mRNA after both 6 weeks and 12 weeks of resistance training (Willoughby, 2004). The difference could be attributed to the timing of sample collection. Our study collected muscle samples 24h after the last training session while Willoughby collected muscle samples only 15 min after an exercise bout. A landmark study by Spiegelman's group found an alternative splice variant of PGC-1alpha (PGC-alpha 4) to be involved in

resistance training and functioned to regulate muscle mass (Ruas et al. 2012). In the present study, we showed a trend that PGC-1alpha 4 protein levels increased upon mechanical stimulation compared with SED and that the traditional PGC-1alpha 1 protein tended to decrease upon resistance training (Figure 5). Ruas et al. measured the mRNA levels of both total PGC-1alpha and the specific isoform 4 and found that resistance training protocol induced significant increase in both total PGC-1alpha and PGC-1alpha 4 gene expressions and that there was a concomitant decrease in myostatin mRNA levels. The decrease of PGC-1alpha 1 protein levels could be explained by increased muscle mass, which resulted in a reduction of PGC-1alpha 1 protein concentration when the total amount of PGC-1alpha 1 protein didn't change.

Another interesting finding in the present study was that mediators involved in muscle protein breakdown seemed to be upregulated upon mechanical stimuli. It was found that no significant differences exist in either the phosphorylated form of FOXO3A or the total FOXO3A protein levels (Figure 6). However, the pilot data showing the ratio of phosphorylated FOXO3A over total FOXO3A did demonstrate a decrease of phosphorylated FOXO3A when normalized to the total protein level, suggesting that there might be an increase in muscle protein breakdown (Figure 6). FOXO3A is a transcriptional factor that can regulate the expressions of two muscle specific E3 ubiquitin ligases, atrogin 1 and MuRF1. Once phosphorylated, FOXO3A will translocate out of the nucleus to be inhibited from its activation on the expressions of the aforementioned proteolytic proteins. Therefore, with a decreased ratio of p-FOXO3A/FOXO3A, one can expect that the expression of MuRF and atrogin would be

upregulated. Current study did show that MuRF was significantly elevated in the CP group when compared to SED and trended to be greater in the other two exercise groups than the SED group (Figure 6). Atrogin was also trending to be higher in the CP and WP group when compared to SED (Figure 6). Resistance exercise is believed to elevate both MPS and MPB without supplementation. In the current exercise protocol, rats were brought to failure in every training session, which might lead to a substantial increase in MPB due to robust elevation of cortisol. Studies have shown that generation of cortisol during high intensity resistance exercise can even lead to a negative net protein balance. It was thought that carbohydrate supplements could attenuate MPB by suppressing cortisol levels while upregulating insulin release. Wang et al. demonstrated that the corticosterone levels were significantly higher in the PLA and WP groups at the end of week 8 than SED group. However, CP group didn't differ in corticosterone levels from SED group, suggesting that carbohydrate might indeed play a positive role in attenuating cortisol levels (unpublished data). The reason as to why MuRF protein expression was higher in CP group could be attributed to that rats in this particular group carried more weight till the end of 8 weeks and might have stimulated MPB more than the other groups.

In summary, consistent with the previous studies using the same exercise protocol (Hornberger and Farrar, 2004), there was an increase in mass of the FHL muscle as well as an increase in myofirillar protein content, suggesting protein accumulation over the 8 weeks of training. Elevation of IGF-1 and reduction of myostatin suggests that protein accretion may be achieved through Akt-mTOR signaling pathway, which serves

as a critical signaling pathway regulating protein synthesis and needs to be further investigated. Increase in MuRF and other related proteolytic markers indicate that there could be an upregulation of protein breakdown. However, since there was a positive net protein balance suggested by muscle hypertrophy, the rate of muscle protein synthesis must have surpassed the rate of muscle protein breakdown. It would be of great significance to obtain direct evidence of muscle protein synthesis and related signaling substrates to further understand the mechanisms in terms of how resistance training and different supplements dictate muscle hypertrophy.

FIGURES

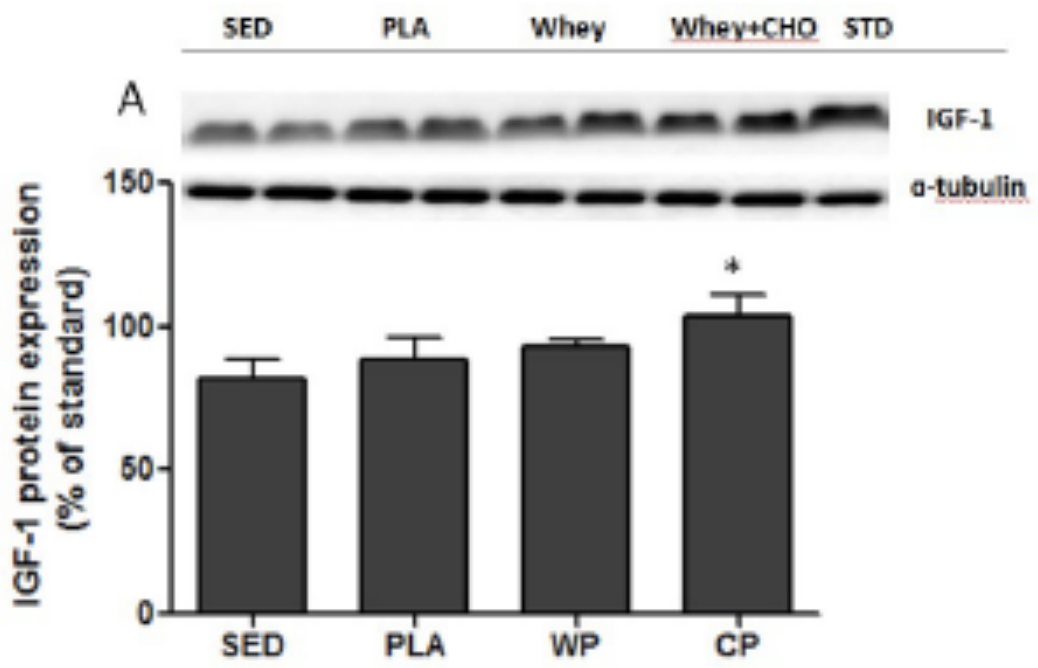


Figure 1: IGF-1 protein expression. Taken from Wang et al. (unpublished).

IGF-1 protein expression as a percentage of a standard sample obtained from an insulin-stimulated tissue. Data are presented as mean ± SEM (n=7-8 per group). *, $p \leq 0.05$ vs SED.

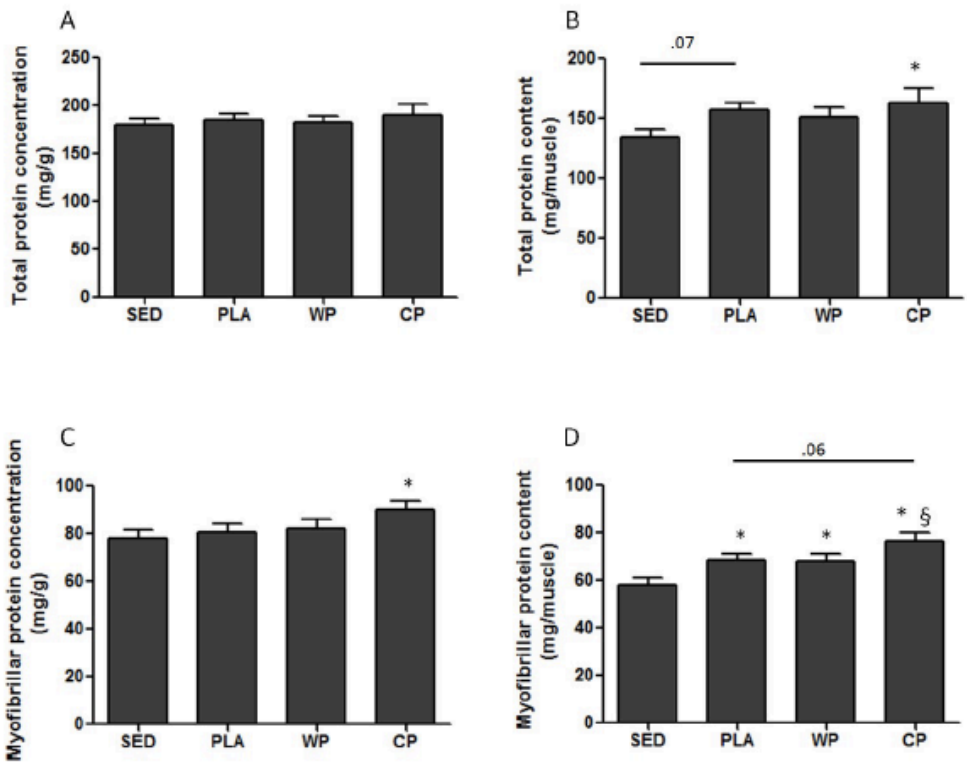


Figure 2: Total and myofibrillar proteins. Taken from Wang et al. (unpublished).

A) Total protein concentration. B) Total protein content per muscle. C) Myofibrillar protein concentration. D) Myofibrillar protein content per muscle. Data are presented as mean ± SEM (n=7-8 per group). *, $p \leq 0.05$ vs SED. §, $p \leq 0.05$ vs WP.

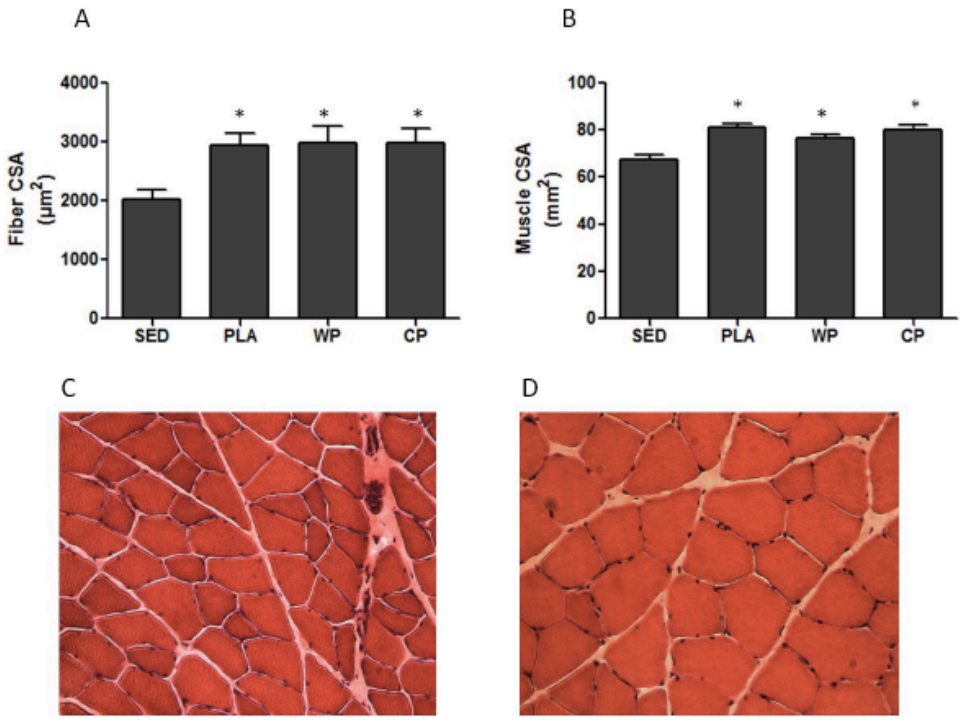


Figure 3: Cross sectional area. Taken from Wang et al. (unpublished).

A) Fiber cross sectional area. B) FHL muscle cross sectional area. C) Cross section of FHL muscle in untrained rats (20x objective lens). Data are presented as mean ± SEM (n=7-8 per group). *, $p \leq 0.05$ vs SED.

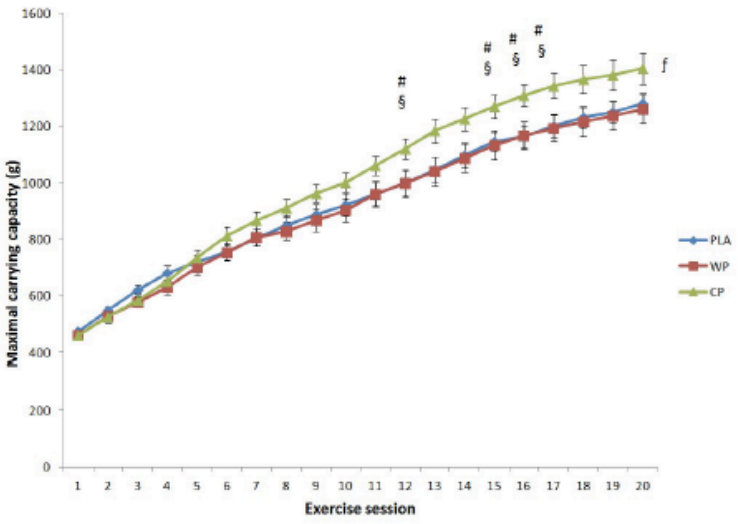
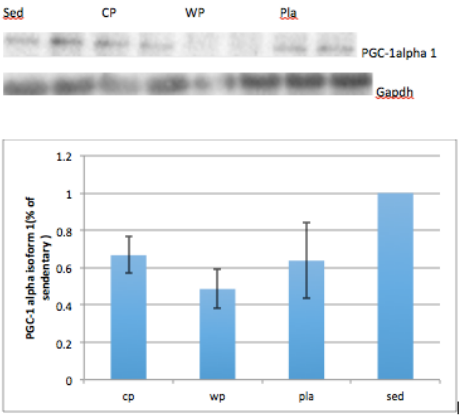
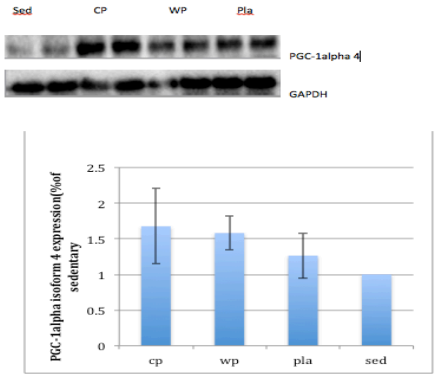


Figure 4: Maximal carrying load per training session over 8 weeks.

Taken from Wang et al. (unpublished). Data are presented as mean ± SEM (n=7-8 per group). *f*, significant treatment effect in CP vs PLA and WP. #, $p \leq 0.05$ vs PLA, §, $p \leq 0.05$ vs WP.



A



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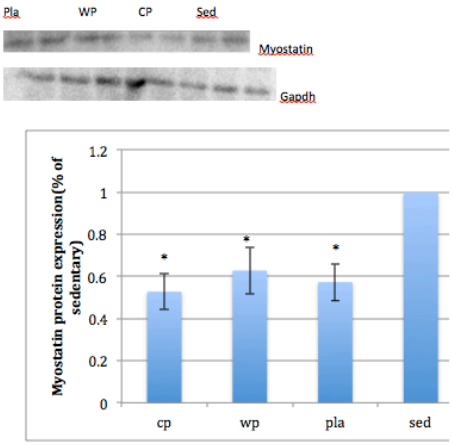
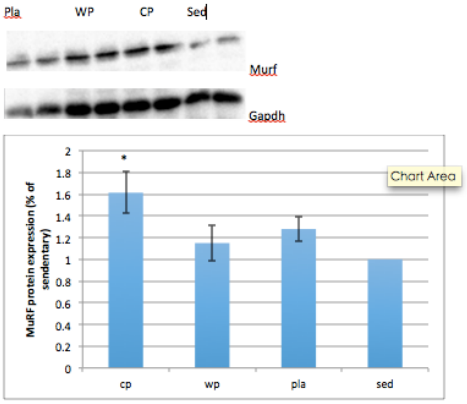


C

Figure 5: Myostatin protein expression decreases after resistance training.

A) PGC-1alpha isoform 1 protein expression expressed as a percentage of sedentary control. B) PGC-1alpha isoform 4 protein expression expressed as a percentage of sedentary control. C) Myostatin protein expression expressed as a percentage of sedentary control. Data are presented as mean ± SEM (n=7-8 per group). *, $p \leq 0.05$ vs SED.



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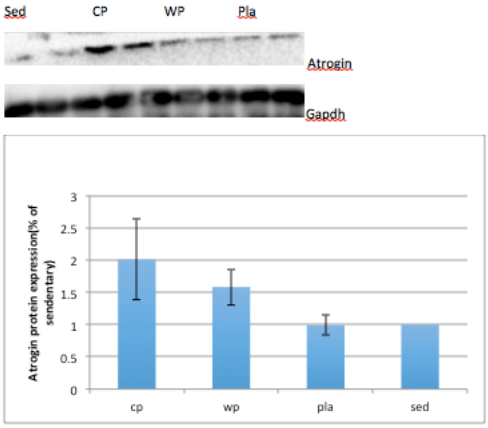

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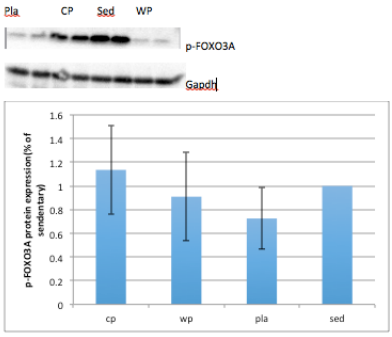

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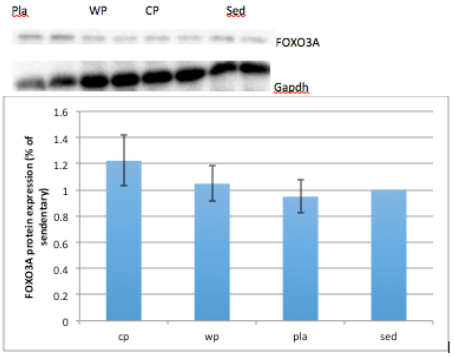


D

Figure 6: MuRF protein expression increases after resistance training when supplemented with CHO+Whey.

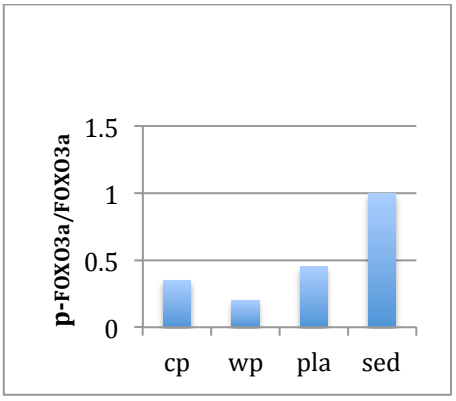
E 

Figure 6: MuRF protein expression increases after resistance training when supplemented with CHO+Whey.

A) MuRF protein expression expressed as a percentage of sedentary control. B) Atrogin protein expression expressed as a percentage of sedentary control. C) p-FOXO3A protein expression expressed as a percentage of sedentary control. D) FOXO3A protein expression expressed as a percentage of sedentary control. E) Pilot data for p-FOXO3A/FOXO3A ratio. Data are presented as mean ± SEM (n=7-8 per group). *, $p \leq 0.05$ vs SED.

Appendices

APPENDIX A: WESTERN BLOT

Solutions

1.5M Tris, pH8.8 (500ml)

Add 90.82g Trisbase to 400ml ddH_2O stirring on a magnetic plate and bring to 500ml volume with ddH_2O. Adjust pH to 8.8 with 12N and 1N HCl. Store it at 4°C.

1.0M Tris, pH 6.8 (500ml)

Add 60.57g Trisbase to 400ml ddH_2O stirring on a magnetic plate and bring to 500ml volume with ddH_2O. Adjust pH to 6.8 with 12N and 1N HCl. Store it at 4°C.

20% SDS (100ml)

Add 20g SDS to 80ml ddH_2O stirring on a magnetic plate and bring to 100ml volume with ddH_2O. Filter this solution through Whatman #1 filter paper.

10% APS (1ml)

Add 0.1g APS to 1.5ml test tube with 1ml ddH_2O and vortex until dissolve. Make fresh daily.

10 x Running Buffer (2L)

Reagent	Amount (g)
Trisbase	60.56
Glycine	288.4
SDS	20g

Add the reagents into 1.5L ddH_2O stirring on a magnetic plate and bring to the volume with ddH_2O

10x TTBS (2L, pH7.4)

Reagent	Amount
TrisBase	24.2 g
NaCl	175.36 g
Tween 20	12 ml

Add the reagents into 1.5L ddH_2O stirring on magnetic plate and bring to the volume

Anode II solution

Component	Reagent and Amount
25mM Tris	25 mL of 1 M Tris Stock
0.05% SDS	2.5 mL of 20% SDS
10% methanol	100 mL
10mM β-mercaptoethanol	0.78 mL

** Bring to 1 L*

Cathode solution
(Bring to 1L)

Component	Reagent and Amount
25mM Tris	25 mL of 1 M Tris Stock
40 mM α-amino hexanoic acid	5.248 g
0.05% SDS	2.5 mL of 20% SDS
10% methanol	100 mL
10mM β-mercaptoethanol	0.78 mL

Procedure

12.5% Separating Gel	**15% Separating Gel**	**18% Separating Gel**
3.13 mL 40% Acrylamide	3.76 mL 40% Acrylamide	4.51 mL 40% Acrylamide
0.35 mL 2% Bis	0.41 mL 2% Bis	0.49 mL 2% Bis
2.50 mL 1.5 M Tris pH 6.8	2.50 mL 1.5M Tris pH 6.8	2.50 mL 1.5M Tris pH 6.8
50 uL 20% SDS	50 uL 20% SDS	50 uL 20% SDS
3.93 mL dH2O	3.23 mL dH2O	2.39 mL dH2O
32.5 uL 10% APS	38.94 uL 10% APS	46.71 uL 10% APS
6.25 uL TEMED	7.50 uL TEMED	8.99 uL TEMED

1.Add all reagents above into 50ml test tube. Then mix them and fill ¾ full caster with a glass pipette. Overlay with 300ul butanol. Allow 1h for gel to polymerize.

2.After gel polymerization, pour off butanol from the resolving gel and rinse between casting plates with ddH_2O and dry it with KimWipes.

3.Preparing stacking gel solution (for 2 gels)

5% Stacking Gel

1.25 mL 40% Acrylamide

0.65 mL 2% Bis

1 mL 1.5 M Tris pH 6.8

50.0 Ul 20% SDS

6.99 mL dH2O

50 uL 10% APS

10 uL TEMED

4.Add al reagents above into 15ml test tube. Then mix them and fill the caster with stacking gel solution then put combs into place. Allow 45 min for stacking gel to polymerize.

5.Preparing samples

a. Take the samples our of -80°C and thaw them on ice

b. Dilute samples 1:3 with sample buffer in labeled microcentrifuge tubes.

c. Vortex and place in boiling water (~90-95°C) for 8min

6.Preparing 1 x Running buffer

Add 100ml 10x Running buffer into 900ml ddH_2O stirring on a magnetic plate.

7.After stacking gel has polymerized, carefully remove the combs and assemble gel apparatus. Fill inner chamber and out chamber with 1 x running buffer.

8.Turn on power supply and set to constant current (50 mA for every module connected), 200 V, and 200 W. Run for appropriate time (i.e. when desired separation is achieved).

9.After running is completed, proceed to membrane transfer.

1.) Pour an appropriate amount of Anode I solution into a weigh boat.

2.) Remove gel from electrophoresis module and carefully remove short plate. Slowly cut gel at desired molecular weights. Notch the top corner of lane 1 and place in Anode I solution filled weigh boat.

3.) Cut a piece of PDVF membrane the size of the gel just prepared. Mark the upper right-hand corner with a pencil. Place this into pure methanol, then move to another weigh boat containing Anode I solution. Allow to equilibrate for at least 5 minutes.

4.) Cut 2 pieces of extra-thick blotting paper that corresponds in size to the membrane (2 pieces for every membrane). Soak 1 of these in Anode II solution.

5.) Open transfer cell and gently place Anode II solution-soaked blotting paper to electrode. Roll over with test tube to remove all air bubbles.

6.) Remove membrane from Anode I solution and place on top of blotting paper. Roll test tube over.

7.) Gently place soaked gel over membrane and make sure notched corner lines up with marked membrane corner. Check to see if any air bubbles.

8.) Soak other piece of blotting paper in Cathode solution. Remove and place over gel. Roll over with test tube.

9.) Replace transfer cell cover. Turn ON power supply and set to 300 mA constant current, 25 V, and 200 W. Time of transfer will depend on size and abundance of protein of interest.

10. After transfer, proceed to immunoblot:

1.) Prepare 25 mL of 5% milk in TBST for every membrane being transferred. Pour milk into plastic shaking dish.

2.) Remove membrane from transfer cell and place in milk. Rotate at room temperature for at least 1 hour.

3.) Depending on primary antibody to be used, prepare 5 mL of either 1% milk-TBST (Santa Cruz antibodies) or 5% BSA-TBST (Cell Signaling antibodies). Transfer to a properly labeled 15 mL conical tube. Add appropriate amount of primary antibody to achieve desired dilution (usually 1:1000 works).

4.) Pour milk out of shaker and add a small amount of TBST to rinse milk off of membrane. Using gloves and forceps, remove membrane from shaker and curve to fit into the tube containing the prepared primary antibody solution. Make sure protein covered side is facing inward (i.e. is exposed to the primary antibody). Place in 4 ºC rotator overnight.

5.) Add ~15 mL of TBST to a plastic shaker, and remove membrane from 15 mL conical tube to place in shaker. Rotate at room temperature for 15 minutes. Perform 3 washes total. Return primary antibody solution to 4 ºC.

6.) Prepare 10 mL of either 1% milk-TBST (Santa Cruz antibodies) or 5% milk-TBST (Cell Signaling antibodies). Add appropriate amount of correct secondary antibody to achieve a 1:2000 dilution (may need to adjust, depending on antibody used). Rotate at room temperature for at least 1 hour (2 hours for better results).

7.) Dump out secondary antibody solution, rinse with TBST, and perform 5 5-minute washes with TBST.

Membrane stripping and re-probing

1. Wash membrane in the ddH2O for 5min.
2. Add membrane to 0.2N NaOH for 5-7 min.
3. Wash membrane in the ddH2O for 5 min, followed by another 5 min wash using TBST.
4. After washing, membrane can be re-blocked and then re-probed with another antibody.

APPENDIX B: RAW DATA

MuRF

cp	wp	pla	sed
1.120006955	0.734638527	0.831860697	1
2.00900583	1.631986775	1.696645518	1
1.99446746	2.011636438	1.568391694	1
2.560282546	1.306386054	1.349275333	1
1.219361056	0.778795959	1.455988754	1
0.976959114	0.938050456	0.972843108	1
1.686947021	0.717075941	1.076927549	1
1.338672815	1.07846249		1

Atrogin

cp	wp	pla	sed
0.707878828	0.769840104	0.76297374	1
0.686343014	1.033614101		1
0.721139902	1.917511866	1.807912917	1
3.231235849	1.403049577	0.99352492	1
1.331263923	0.966618679	0.663594351	1
5.033127936	2.745865683	0.782227654	1
2.357105538	2.240965077	0.946443942	1

Myostatin

cp	wp	pla	sed
0.826002747	0.812038277	0.807463289	1
0.305787216	0.38249576	0.240144133	1
0.351463893	0.283639069	0.597576414	1
0.576286718	0.792040773	0.701895541	1
0.574163761	0.866086592	0.515230201	1

FOXO3A

cp	wp	pla	sed

1.396256191	1.367060908	0.663559403	1
0.898109821	1.092107592	1.44112084	1
0.980289524	1.015689274	0.545670085	1
0.687119716	0.653182415	1.482842456	1
0.613551131	0.584628493	0.917372931	1
0.925325585	0.578545775	0.613360481	1
0.696030779	0.811881064	0.955043387	1
2.175139381	1.465184545	0.455965923	1
0.97580735	1.786927151	0.548359338	1
1.499464916	0.575533399	1.274771299	1
2.604099805	1.59599372	1.539370429	1

p-FOXO3A

cp	wp	pla	sed
0.833131558	0.123140885	1.226787976	1
0.475871932	0.452177635	0.180104413	1
2.207873891	1.369117004	1.102878382	1
1.027913085	1.694953831	0.393556059	1

p-FOXO3A/FOXO3A

cp	wp	pla	sed
0.383024447	0.151673552	0.717697378	1
0.317361164	0.253047605	0.188582441	1

PGC-1alpha 4

cp	wp	pla	sed
1.139567702	1.274946274	1.355793673	1
0.648871712	1.082552889	0.372875381	1
1.829451727	2.002934512	1.870807041	1
3.084392518	1.966231161	1.458852521	1

PGC-1alpha 1

cp	wp	pla	sed	
0.597270562	0.55663121	0.648019729		1
0.456591331	0.185356953	0.385304465		1
0.919510598	0.547114965	0.314651877		1
0.695572024	0.655357654	1.203460069		1

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